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Research Article

Micronuclei and Other Erythrocyte Nuclear Abnormalities in Fishes from the Great Lakes Basin, USA

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Biological markers (biomarkers) sensitive to genotoxic and mutagenic contamination in fishes are widely used to identify exposure effects in aquatic environments. The micronucleus assay was incorporated into a suite of indicators to assess exposure to genotoxic and mutagenic contamination at five Great Lakes Areas of Concern (AOCs), as well as one non-AOC (reference) site. The assay allowed enumeration of micronuclei as well as other nuclear abnormalities for both site and species comparisons. Erythrocyte abnormality data was also compared to skin and liver tumor prevalence and hepatic transcript abundance. Erythrocyte abnormalities were observed at all sites with variable occurrence and severity among sites and species. Benthic-oriented brown bullhead

(*Ameiurus nebulosus*) and white sucker (*Catostomus commersonii*) expressed lower rates of erythrocyte abnormalities, but higher rates of skin and liver neoplasms, when compared to pelagic-oriented largemouth bass (*Micropterus salmoides*) or smallmouth bass (*Micropterus dolomieu*) at the same site. The reduced erythrocyte abnormalities, increased transcript abundance associated with Phase I and II toxicant responsive pathways, and increased neoplastic lesions among benthic-oriented taxa may indicate the development of contaminant resistance of these species to more acute effects. Environ. Mol. Mutagen. 00:000–000, 2017. © 2017 This article is a U.S. Government work and is in the public domain in the USA. Environmental and Molecular Mutagenesis published by Wiley Periodicals, Inc. on behalf of Environmental Mutagen Society

Key words: erythrocyte micronuclei; nuclear abnormalities; white sucker; brown bullhead; bass

INTRODUCTION

Areas of Concern (AOCs) are defined by the U.S.–Canada Great Lakes Water Quality Agreement, Annex 2 of the 1987 Protocol as “geographic areas that fail to meet the general or specific objectives of the agreement where such failure has caused or is likely to cause impairment of beneficial use of the area’s ability to support aquatic life” [International Joint Commission (IJC), 1989]. One of the 14 beneficial use impairments (BUIs) recognized at AOCs is “fish tumors and other deformities”. The Great Lakes Restoration Initiative (GLRI), a multiagency effort that began in 2010, specifically targets certain priorities, one of which is evaluating and monitoring progress in improving conditions within AOCs (http://glri.us/pdfs/glri_actionplan.pdf). Historically, legacy sediment contaminants such as polychlorinated biphenyls (PCBs) and polynuclear aromatic hydrocarbons (PAHs) have been the focus of concern at AOCs and most Remedial Action Plans (RAPs) list PAHs and/or PCBs as the likely cause of the fish tumor and other BUIs. Hence, significant resources have been directed at dredging,

removing and remediating contaminated sediment (www.epa.gov/glnpo/sediment/realizing2/RR2report.PDF).

Chemicals of emerging concern (CEC) are increasingly detected within the Great Lakes watershed, however there

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is much less information regarding their hazards and effects. The co-occurrence of legacy contaminants and CECs in complex mixtures led to the conclusion that “effects-based monitoring in the context of multiple stressors should be developed and implemented to supplement the current chemical monitoring regimes” (Great Lakes Chemicals of Emerging Concern Advisory Work Group, 2009, <http://www.ijc.org/files/publications/C220.pdf>). In 2010, a multi-agency research program, funded through the GLRI, was initiated to conduct exploratory and proof-of-concept studies for adverse effects-based monitoring and surveillance [Ekman et al., 2013]. One component of this program was the assessment of wild populations present at potentially impacted sites (AOCs). A suite of biomarkers ranging from organism to molecular-level was developed for multiple fish species. These included well-established indicators of exposure to chemical contaminants including endocrine disruption and neoplasia [Blazer et al., 2014a] as well as development of transcriptome analyses for the non-model wild fish species studied [Hahn et al., 2016]. Micronuclei (MN) and other nuclear abnormalities (NA) of erythrocytes, widely used as indicators of exposure to genotoxic and mutagenic contaminants in fish species [Al-Sabt and Metcalfe, 1995; Bolognesi et al., 2006; Bolognesi and Hayashi, 2011; Obiakor et al., 2012] were assessed as possible additions to the suite of necropsy-based, histopathological, serological and molecular indicators currently utilized.

Micronuclei and NA form during the proliferative phase of the cell cycle in numerous cell types. A micronucleus forms during telophase of cell division when either whole or fragmented chromosomes become encapsulated in a nuclear envelope and assume the properties of an interphase nucleus which is dramatically reduced in size [Al-Sabt and Metcalfe, 1995]. This can occur due to either clastogenicity (chromosomal breaking) or aneugenicity (mitotic spindle dysfunction) resulting from exposure to a genotoxic contaminant. The assay also specifically evaluates individual cells for the presence of NA which include binucleated cells, lobed, notched or blebbed (nuclear buds) nuclear membranes. The exact pathways leading to the induction of NA are less understood, but have also been used as indicators of exposure to mutagenic or genotoxic contaminants in numerous fish species [Baršienė et al., 2006; da Rocha et al., 2011]. A variety of chemicals such as PCBs, PAHs, heavy metals and pesticides [Al-Sabt and Metcalfe, 1995; Bolognesi and Hayashi, 2011; Obiakor et al., 2012] and more recently CEC [Teles et al., 2004; Filby et al., 2007; Iturburu et al., 2016] have been linked to erythrocyte MN and NA induction in various fishes.

The species evaluated in this study were largemouth (LMB) *Micropterus salmoides* and smallmouth (SMB) *M. dolomieu* bass, predominately pelagic, higher trophic level species, and brown bullhead (BB) *Ameiurus nebulosus* and white sucker (WS) *Catostomus commersonii*, representing

the more benthic, lower trophic level species. Brown bullhead and WS have been used extensively throughout the Great Lakes to assess the “fish tumor and other deformities” beneficial use impairment at AOCs [Rafferty et al., 2009; Simmons et al., 2012; Blazer et al., 2017]. Both bass species have been shown to be sensitive species for assessment of exposure to endocrine modulating and other CEC [Hinck et al., 2009; Blazer et al., 2012; Iwanowicz et al., 2016]. Our specific objectives were to 1) explore the utility of MN and NA in Great Lakes indicator species, 2) compare MN and NA prevalence and severity among species and sites and 3) evaluate the relationship between these biomarkers, fish tumor prevalence and hepatic transcript abundance.

MATERIALS AND METHODS

Site Information

Fish were collected from sites located within five AOCs and one non-AOC reference site in spring (Fig. 1). The AOC sites included the lower Ashtabula River located near Ashtabula, Ohio; the Trenton channel of the Detroit River sampled south of Detroit, Michigan; the lower Genesee River sampled near Rochester, New York; the lower Milwaukee River sampled near Milwaukee, Wisconsin and St. Louis Bay sampled near Duluth, Minnesota. Conneaut Creek, near Conneaut, Ohio, has been previously used as a reference site for comparison with the nearby Ashtabula River AOC [Iwanowicz et al., 2012]. In fall, fish were collected at the Ashtabula and Conneaut sites for a seasonal comparison.

Field Collection

The target sample size was 20 mature adults (3 years or older) of each of two species at each site. Fish were collected by electrofishing and/or fyke nets. Spring sampling occurred prespawn and during spawning between April 12 and June 21, 2011. Fall sampling occurred October 4–5, 2011. All fish were euthanized with Finquel (MS-222; Argent Chemical Co., Redmond, WA), 300 mg/L at pH 7.0. Fish were weighed to the nearest gm, measured (total length in mm) and a peripheral blood sample was extracted from the caudal vein using a heparinized syringe with 23 gauge needle (Fisher Scientific, Pittsburgh, PA). A drop of blood was immediately placed on duplicate clean glass microscope slides, drawn across the surface and allowed to air-dry. A necropsy-based health assessment was conducted on each fish and pieces of any external abnormalities were preserved in Z-fixTM tissue fixative (Anatech LTD, Battle Creek, MI). At least five pieces of liver from each fish were also preserved in Z-fixTM for subsequent evaluation of microscopic pathology. Small sections of liver were preserved in RNAlaterTM Stabilization Solution (ThermoFisher) for transcript abundance analysis.

Evaluation of Erythrocyte Micronuclei and Nuclear Abnormalities

Slides were fixed in absolute methanol for 10 minutes within four hours of initial preparation. Slides were stained with Giemsa solution (Fluka Analytical, St. Louis, MO), 1:12:2 (w/w/w) in glycerol/methanol 5:24 (v/v) for 45 minutes, followed by two, 45 minute distilled water baths. Cover slips were mounted on stained slides and slides evaluated by light microscopy at 600× magnification. A minimum of 1,000 erythrocytes were evaluated for each fish by scoring abnormalities of at least 200 erythrocytes in five stratified random locations on each slide [Al-Sabt and Metcalfe, 1995].

Nuclear abnormalities were identified as defined by Carrasco et al. [1990]. Briefly, a micronucleus is defined as a round cytoplasmic

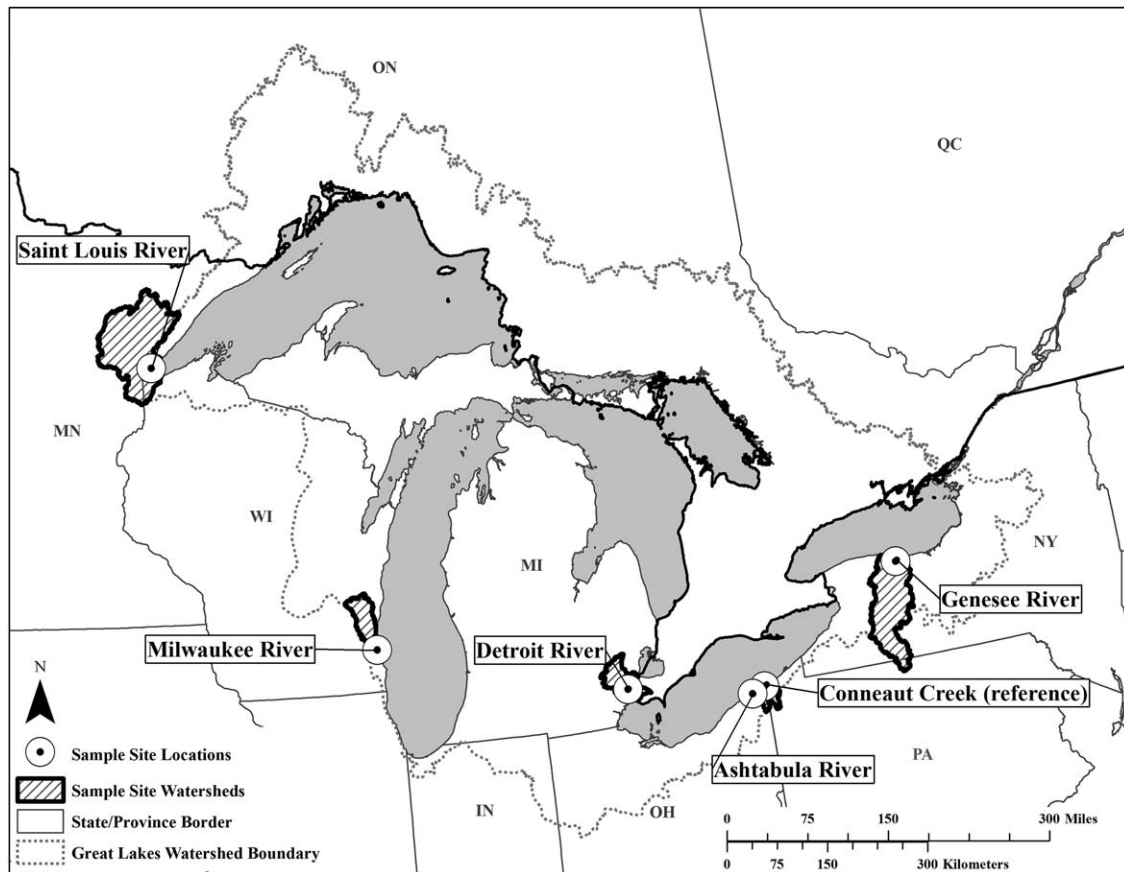


Fig. 1. Sample sites throughout the Great Lakes basin.

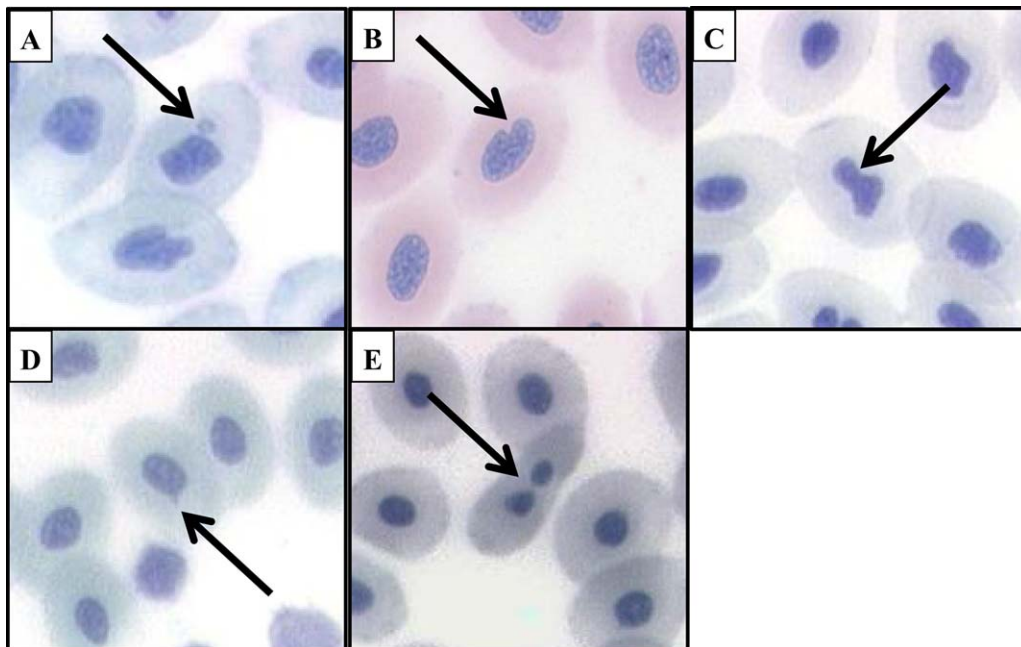


Fig. 2. Erythrocyte abnormalities. (A) Micronucleus (arrow), note the "lobed" nucleus at the bottom of the pane B. Notched nucleus (arrow). (C) Lobed nucleus (arrow), note the notched nucleus within the partial erythrocyte at the bottom of the pane. (D) Blebbed nucleus (arrow). E. Binucleated cell (arrow). All pictures were taken at 600× magnification.

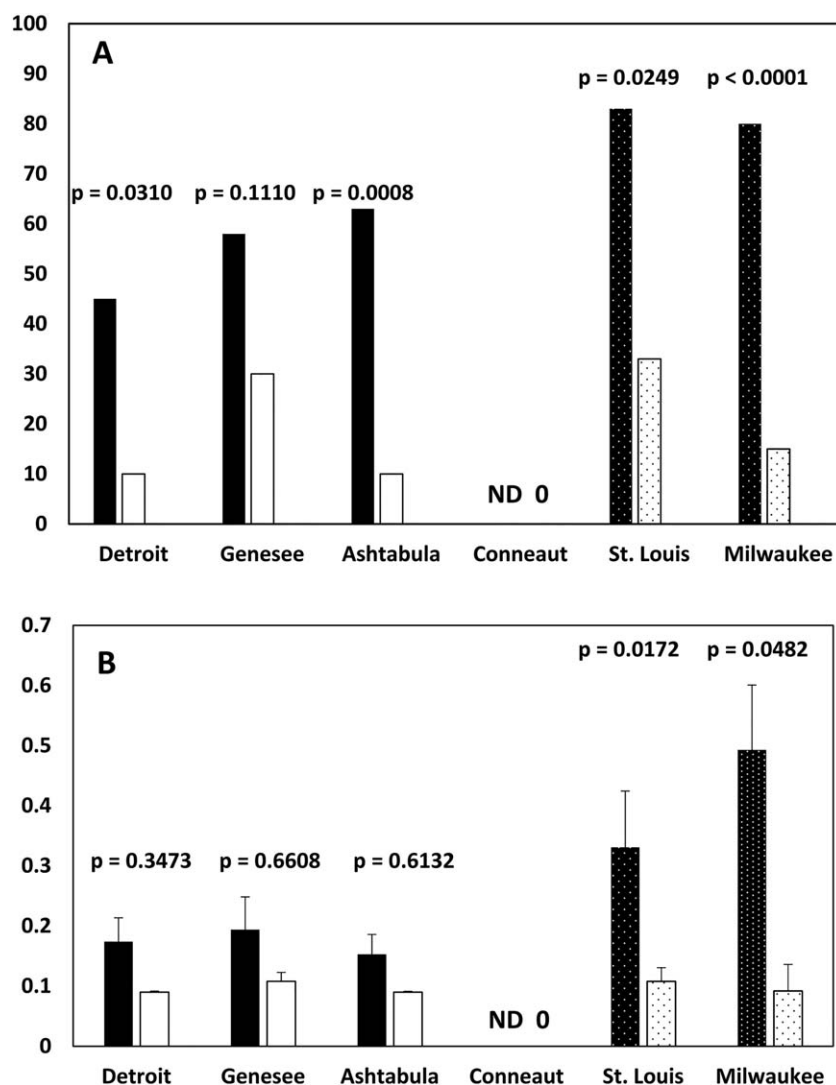


Fig. 3. (A) Percent of individual fish at a site having any micronuclei. (B) Mean severity or percentage of micronuclei per individual of those fish with micronuclei. Black bars represent largemouth bass; white bars represent brown bullhead; black bars/white dots represent smallmouth bass; white bars/black dots represent white sucker. ND = no data. *P* value indicates difference between the species at a site.

inclusion having a diameter one-tenth to one-third that of the primary nucleus (Fig. 2A). Notched nuclei have clear slits that extend well into the nuclear envelope (Fig. 2B). Lobed nuclei have large evaginations of the nuclear envelope that have no clear shape or definition (Fig. 2C). Blebbed nuclei or nuclear buds have small evaginations of the nuclear envelope resembling a micronucleus, however are attached by a small threadlike stalk (Fig. 2D). A binucleated cell contains two nuclei that are not attached and relatively similar in size (Fig. 2E). Results are presented in three ways: (1) the prevalence or percent of individuals collected at a site expressing MN or NA, (2) the severity or mean number of MN or NA in those individuals, and (3) the site severity or mean number of MN or NA in all individuals of a given species at that site.

Molecular Analysis

A hepatic transcript abundance assay was recently developed for use in these four species of fishes [Hahn et al., 2016]. A subset of genes related to legacy contaminant exposure/metabolism and oxidative

damage was chosen for comparison of transcript abundance with the MN and NA occurrence. The genes selected and species in which each was investigated were: aryl hydrocarbon receptor (*ahr*; all species), cytochrome P4501A (*cyp1a*; BB, LMB, SMB), cytochrome P4503A (*cyp3a*; BB, LMB, SMB), glutathione peroxidase (*gpx*; all species), glutathione S transferase (*gst*; all species), heat shock protein 70 (*hsp70*; all species), heat shock protein 90 β (*hsp90 β* ; all species), proliferating cell nuclear antigen (*pcna*; BB, WS), and superoxide dismutase (*sod*; all species). The nCounter analysis[®] (NanoString Technologies, Seattle, WA) assay was used to identify transcript abundance in liver tissue preserved in RNAlater[®]. Briefly, transcriptome databases for each species were utilized in the creation of custom CodeSet designed by Nanostring Technologies. Abundance analysis was performed on the Nanostring Technologies platform at the University of Pittsburgh Genomics and Proteomics Core Laboratory (Pittsburgh, PA). Quality control and normalization of transcript abundance data was performed using nSolver Analysis Software (v2.0). Housekeeper genes were used to normalize count data and were selected to span average counts and % coefficient of variation (CV) values of data for each species. In all species RNA-

binding motif protein X-linked 2 (*rbmx2*), ribosomal protein L8 (*rpl8*) and elongation translation initiation factor 3D (*eltf3d*) were included as housekeeper genes. Additional housekeeper genes included phosphoenolpyruvate carboxykinase (*pepck*), TATA box binding protein (*tbp*) and β -actin (*Bact*) in SMB; β act, elongation factor 1 α (*efl1*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) in LMB; *efl1* and *hprt1* in BB; and *pepck* and MUS81 structure-specific endonuclease subunit (*mus81*) in WS.

Neoplastic Lesions of Liver and Skin

Methodology and prevalence of skin (papilloma and squamous cell carcinoma) and liver (hepatocellular adenoma and carcinoma, cholangioma and cholangiocarcinoma) tumors in BB [Blazer et al., 2014b] and WS from the St. Louis River [Blazer et al., 2014c] were previously published. The prevalence of these tumors at the Milwaukee site has not been previously reported but methods were the same as described in Blazer et al. [2014c].

Statistical Analysis

The Kruskal-Wallis and Wilcoxon rank sum tests were performed to determine statistically significant differences for MN/NA prevalence and severity between species and among sites. The Wilcoxon rank sum test was also used to determine statistical differences within species, among season. A chi-squared test was conducted to determine significant differences within sites, among species; as well as within species, among erythrocyte and histological endpoints. For all applicable tests, significance was determined at $\alpha = 0.05$. All statistical analysis was performed using the R statistical package [R Core Development Team, 2016].

Selected genes were compared within species and among sites using the Kruskal-Wallis test using MultiExperiment Viewer [Howe et al., 2011]. MN/NA severity and transcript abundance was compared using Spearman's rank correlation coefficient. These analyses were performed using the R statistical package [R Core Development Team, 2016]. Significance was determined at $\alpha \leq 0.05$.

RESULTS

Information on age, morphological (length, weight, condition factor) and necropsy-based findings for all fish can be found in Blazer et al. [2014a].

Site Comparison: Micronuclei

Site comparisons were conducted with each of the four species collected in the spring. The prevalence of BB with MN was significantly higher at Genesee as compared to Conneaut while BB collected at Detroit and Ashtabula were intermediate and not significantly different than the other sites (Fig. 3A). There was no difference in the mean severity of MN in those positive fish (Fig. 3B). When comparing the site severity (all fish), the Genesee had the highest MN severity, which was significantly higher than both the Ashtabula and Conneaut while the Detroit was intermediate (Table I). White sucker were collected at two sites. There was no significant difference in MN prevalence or severity (Fig. 3) or mean site severity (Table I) between the St. Louis and Milwaukee sites.

Largemouth Bass were collected at three sites in the spring. Those from Detroit had the lowest prevalence of

TABLE I. Site Comparisons of Severity of Micronuclei or Other Nuclear Abnormalities

Species and Site ^a	Sample size	Micronuclei	Other Nuclear Abnormalities
<i>Brown Bullhead</i>			
Detroit River	20	0.009 \pm 0.006 ^{bb}	0.227 \pm 0.087 ^{bc}
Genesee River	20	0.033 \pm 0.012 ^b	0.009 \pm 0.006 ^b
Ashtabula River	20	0.005 \pm 0.003 ^b	0.239 \pm 0.071 ^c
Conneaut Creek	20	0 ^b	0.046 \pm 0.024 ^{bb}
<i>White Sucker</i>			
St. Louis River	41	0.038 \pm 0.009 ^b	0.042 \pm 0.012 ^b
Milwaukee River	20	0.014 \pm 0.008 ^b	0.063 \pm 0.025 ^b
<i>Largemouth Bass</i>			
Detroit River	20	0.079 \pm 0.026 ^b	3.075 \pm 0.669 ^b
Genesee River	19	0.113 \pm 0.038 ^b	3.574 \pm 0.409 ^b
Ashtabula River	19	0.097 \pm 0.027 ^b	3.168 \pm 0.710 ^b
<i>Smallmouth Bass</i>			
St. Louis River	6	0.276 \pm 0.094 ^b	2.652 \pm 1.387 ^b
Milwaukee River	20	0.394 \pm 0.097 ^b	2.066 \pm 0.429 ^b

^aData is presented as mean percent (affected cells/total cells evaluated) \pm standard error for all fish.

^bMeans with different letters indicate significant differences among sites for the same species.

MN, however there were no significant differences in prevalence (Fig. 3A), severity of MN positive bass (Fig. 3B) or the site severity means (Table I). Smallmouth bass were collected at two sites, St. Louis and Milwaukee, and MN prevalence were similar (Fig. 3A). Although the severity of those with MN was higher at Milwaukee than at St. Louis, they did not differ significantly (Fig. 3B). The mean site severity was not significantly different between the sites (Table I).

Site Comparison: Nuclear Abnormalities

The pattern of NA prevalence and severity in BB among the four sites was different than the pattern observed for MN. The highest prevalence of NA among BB was observed at Ashtabula which was significantly higher than the prevalence at both Conneaut and Genesee. Prevalence of NAs was significantly higher at the Detroit as compared to the Genesee (Fig. 4A). The severity of those NA-positive fish was highest at Detroit which was significantly higher than at Conneaut and Genesee (Fig. 4B). The mean site severity was highest at the Ashtabula and Detroit, intermediate at Conneaut and lowest at Genesee (Table I). There was no significant difference in the percentage of WS with NA (Fig. 4A), the severity (Fig. 4B) or site severity (Table I) between St. Louis and Milwaukee.

Nuclear abnormalities were observed in both LMB and SMB at all sites (Fig. 4A). There was some variation among sites in severity (Fig. 4B) although not significant, nor were there site severity differences (Table I).

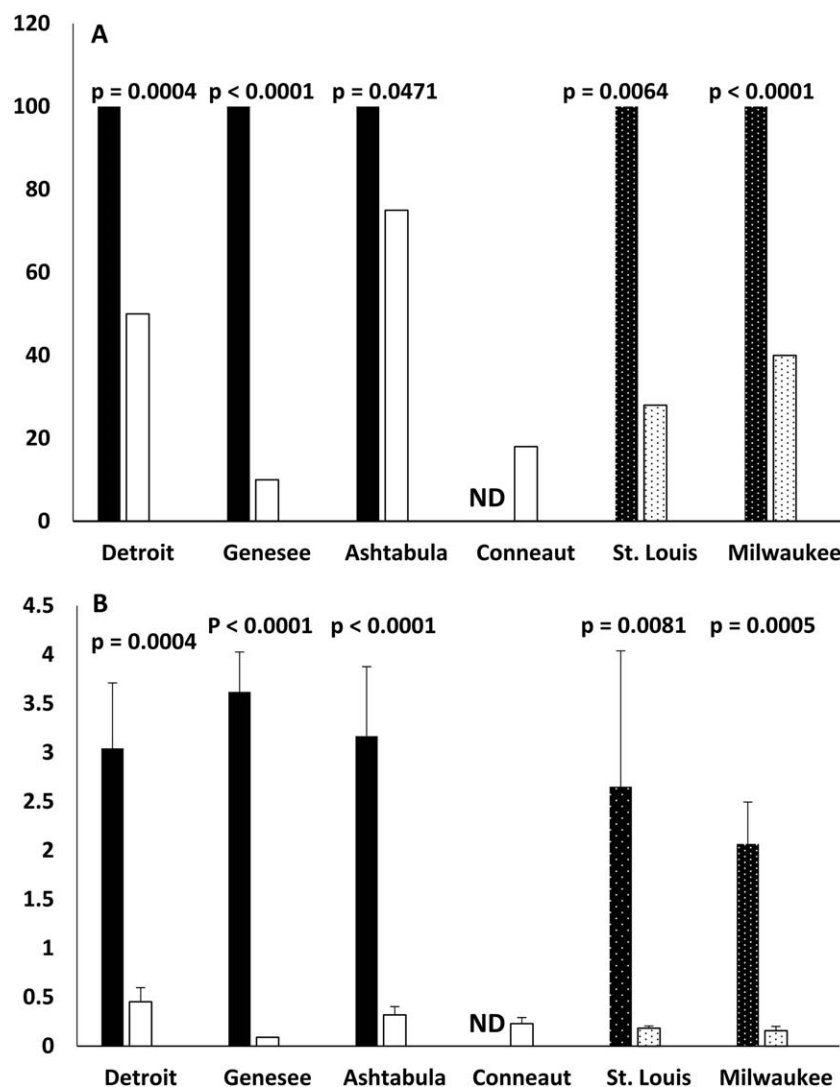


Fig. 4. A. Percentage of individual fish at a site having other nuclear abnormalities. B. Mean severity or percentage of nuclear abnormalities per individual of those fish with micronuclei. Black bars represent largemouth bass; white bars represent brown bullhead; black bars/white dots represent smallmouth bass; white bars/black dots represent white sucker. ND = no data. *P* value indicates difference between the species at a site.

Seasonal Comparison

Brown bullhead were collected at both the Ashtabula River and Conneaut Creek in spring and fall, while LMB were only collected at the Ashtabula during both seasons. There were no significant seasonal differences at either site or species for either MN or NA (Table II).

Erythrocyte Species Comparison

A species-based comparison of BB and LMB MN and NA was evaluated at the Detroit, Genesee and Ashtabula. At all sites, LMB had higher prevalence of MN. This difference was statistically significant for MN prevalence at Detroit and Ashtabula, but not Genesee (Fig. 3A). There was no difference between BB and LMB MN severity among the MN-positive fish (Fig. 3B). At all sites, LMB

had significantly higher NA prevalence (Fig. 4A) and greater severity (Fig. 4B).

A species comparison was evaluated between SMB and WS at Milwaukee and St. Louis sites. At both sites, SMB expressed MN at a significantly higher prevalence and greater severity than WS (Fig. 3). Significantly higher NA prevalence and NA severity of SMB were also observed (Fig. 4).

Association with Skin or Liver Neoplasms

Although erythrocyte abnormalities occurred in both bass species, neoplasms were rarely observed. No skin or liver neoplastic lesions were observed in either LMB or SMB collected in spring. Conversely, skin and liver neoplasms were observed in both BB and WS. The prevalence

TABLE II. Seasonal Comparison of Micronuclei (MN) and Other Nuclear Abnormalities (NA)

	Ashtabula Spring	Ashtabula Fall	Conneaut Spring	Conneaut Fall
Brown Bullhead	<i>N</i> = 20	<i>N</i> = 19	<i>N</i> = 20	<i>N</i> = 20
Prevalence MN	10%	0%	0%	0%
Severity MN	0.009 ± 0.001	0	0	0
MN Site Severity	0.005 ± 0.003	0	0	0
Prevalence NA	75%	47%	20%	15%
Severity NA	0.319 ± 0.085	0.290 ± 0.061	0.171 ± 0.044	0.091 ± 0.001
NA Site Severity	0.239 ± 0.071	0.137 ± 0.044	0.046 ± 0.024	0.014 ± 0.007
Largemouth Bass	<i>N</i> = 19	<i>N</i> = 20		
Prevalence MN	63%	50%		
Severity MN	0.153 ± 0.033	0.190 ± 0.046		
MN Site Severity	0.097 ± 0.027	0.095 ± 0.032		
Prevalence NA	100%	100%		
Severity NA	3.168 ± 0.710	2.316 ± 0.375		
NA Site Severity	3.168 ± 0.710	2.316 ± 0.375		

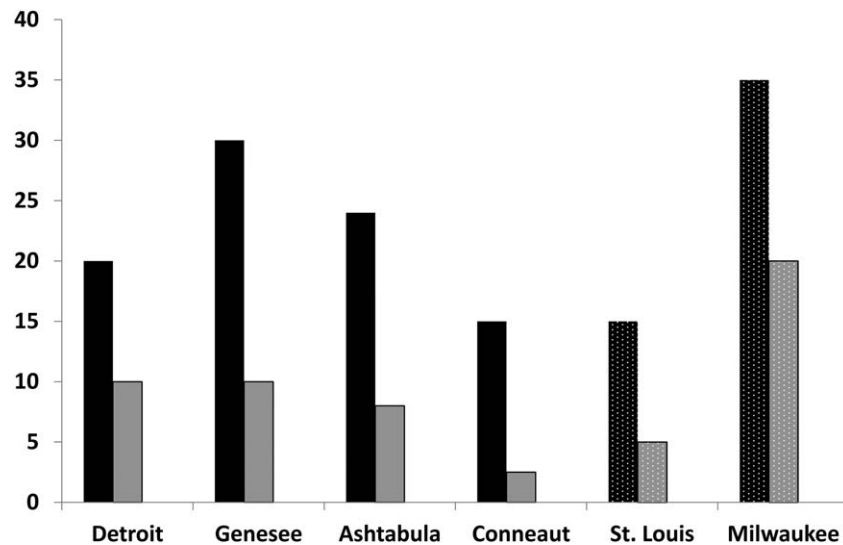


Fig. 5. Percentage of brown bullhead (solid bars) and white sucker (patterned bars) with neoplastic lesions. Black bars represent skin neoplasms and gray bars represent liver (both hepatic and bile duct) neoplasms.

of skin and liver tumors in both species showed a similar site pattern with a higher number of skin versus liver neoplasms observed. Conneaut had the lowest percent of BB with neoplasms, followed by Ashtabula, Detroit and Genesee (Fig. 5). White sucker were collected at Milwaukee, which had the highest number with neoplasms, and St. Louis Bay. There were no statistically significant correlations between MN or NA and skin or liver tumor prevalence for either BB or WS.

Molecular Analysis

There was considerable individual variability in transcript abundance counts. There were some site differences (Tables III and IV), however the more noticeable differences were between species. At sites where BB and LMB were collected, *cyp3a*, *gst*, *hsp70*, *hsp90β*, and *sod*

transcripts were significantly more abundant ($P < 0.001$) in BB. At sites where SMB and WS were collected, higher relative expression ($P < 0.001$) was observed in WS for *ahr*, *gpx*, *gst*, *hsp90β*, and *sod*, while *cyp3a* was higher in SMB. We recognize that differences in amplification of gene regions and varying normalization methods may influence species comparisons.

Association among Erythrocyte and Molecular Endpoints

Correlations between severity of either MN or NA and molecular endpoints were performed by species. There were no strong correlations, although some were statistically significant. In WS, MN severity was positively correlated to *pcna* (Spearman $r = 0.2562$, $P = 0.0463$) while there were no correlations between NA severity and any gene transcripts. For BB NA severity was correlated with

TABLE III. Comparison of Hepatic Transcript Abundance Counts of Benthic Fishes Collected in the Spring^a

Site	<i>ahr</i>	<i>cyp1a</i>	<i>cyp3a</i>	<i>gpx</i>	<i>gst</i>	<i>hsp70</i>	<i>hsp90 β</i>	<i>pcna</i>	<i>sod</i>
Brown Bullhead									
Detroit River	250 ^b (101–477)	8491 ^b (4537–15001)	3535 ^b (2131–5628)	2932 ^c (1790–4445)	11162 ^b (999–25468)	612 ^{bb} (206–956)	12540 ^b (9040–19751)	1122 ^b (331–3421)	5994 ^b (2165–9606)
Genesee River	283 ^b (170–477)	2271 ^b (471–6233)	3307 ^b (1976–4284)	2086 ^b (1470–2879)	11576 ^b (4362–24786)	376 ^b (157–616)	12026 ^b (8985–17923)	292 ^b (117–514)	4067 ^b (2134–5836)
Ashtabula River	228 ^b (110–385)	4288 ^b (984–10058)	3175 ^b (1319–4713)	2162 ^b (1419–3305)	22203 ^b (7469–55566)	759 ^b (246–1237)	13739 ^b (9190–18048)	1161 ^b (258–3334)	6430 ^b (3100–10010)
Conneaut Creek	558 ^b (212–1464)	2793 ^b (1213–7552)	1410 ^b (448–2630)	814 ^b (98–1937)	9347 ^b (1633–18949)	603 ^{bb} (104–1244)	12533 ^b (7930–20091)	468 ^b (13–3712)	6439 ^b (2486–10219)
White Sucker									
St Louis River	89 ^b (24–349)	-	359 ^b (50–754)	4405 ^b (517–10971)	13911 ^b (3409–30639)	-	7208 ^b (2980–13637)	69 ^b (11–159)	2628 ^b (849–5326)
Milwaukee River	63 ^b (21–118)	-	247 ^b (51–489)	3059 ^b (685–6487)	8615 ^b (1677–26267)	-	9917 ^b (5496–14878)	53 ^b (3–168)	2073 ^b (938–4060)

^aData presented as mean counts (range).^bMeans with different letters indicate a significant differences between sites for a particular species.TABLE IV. Comparison of Hepatic Transcript Abundance in Bass Species Collected in the Spring^a

Site	<i>ahr</i>	<i>cyp1a</i>	<i>cyp3a</i>	<i>gpx</i>	<i>gst</i>	<i>hsp70</i>	<i>hsp90β</i>	<i>sod</i>
Largemouth Bass								
Detroit River	64 ^b (17–102)	8169 ^b (560–15495)	2028 ^b (385–3307)	182 ^b (101–248)	8900 ^b (564–12537)	38 ^b (3–126)	4810 ^b (2393–7358)	514 ^b (121–786)
Genesee River	64 ^b (30–105)	3890 ^b (365–11099)	1478 ^b (434–2471)	197 ^b (102–386)	8499 ^b (4106–14492)	51 ^b (1–122)	5332 ^b (2904–8829)	521 ^b (187–1164)
Ashtabula River	73 ^b (19–110)	4442 ^b (1057–8600)	1894 ^{bb} (959–2728)	219 ^b (86–380)	9712 ^b (6990–14905)	51 ^b (7–167)	4782 ^b (3048–8023)	560 ^b (309–944)
Smallmouth Bass								
St Louis River	46 ^b (28–75)	4329 ^b (2280–7178)	1752 ^b (1198–2810)	279 ^b (168–416)	6042 ^b (2943–8636)	56 (6–265)	4092 ^b (2833–5864)	581 ^b (356–893)
Milwaukee River	35 ^b (12–71)	3955 ^b (1226–5941)	1059 ^b (338–2176)	205 ^b (73–380)	5753 ^b (2265–12400)	12 (2–32)	4367 ^b (2434–7939)	838 ^b (459–1325)

^aData presented as mean counts (range).^bMeans with different letters indicate significant differences between sites for a particular species.

pcna (Spearman $r = 0.2320$, $P = 0.0122$). For bass species, NA severity of LMB negatively correlated with *hsp90β* (Spearman $r = -0.32$, $P = 0.0041$), while no significant correlations were noted with MN severity. For SMB, MN severity and *gst* were negatively correlated (Spearman $r = -0.4892$, $P = 0.0112$) and no associations with NA severity were observed.

DISCUSSION

The results of this study emphasize the benefits of assessing multiple fish species, as well as biomarkers at various levels of organization to identify adverse effects and better document the health of aquatic ecosystems. Although our sites were generally distributed among impaired locations with complex mixtures of chemicals, the results suggest differences in chemical composition may occur. The Great Lakes AOCs are within areas of

high human population with industrial sources. Legacy contaminants such as PCBs, PAHs and heavy metals were historically identified as potential causes for many of the BUIs (Table V). Unfortunately concentrations of most legacy contaminants were not measured in water, sediment or tissue during this study. A suite of 134 CECs was measured at the AOC sites around the time of fish sampling. Alkylphenols (9), flavors/fragrance (8), hormones (17), pesticides (11), pharmaceuticals (51), plasticizers/flame retardants (9) and PAHs (9) were included [Lee et al., 2012]. Choy et al. [2017] compared detections of these compounds among the sites sampled. Many of the compounds were below detection or estimated values as they were below the laboratory reporting value. Additionally, the St. Louis River sites were sampled in the summer rather than spring, making identification of relevant compounds difficult. Concentrations of alkylphenols were all below detection or estimated values and hence

TABLE V. Chemical Contaminants of Concern or Identified at Fish Sampling Sites

	Detroit	Genesee	Ashtabula	St. Louis	Milwaukee
Legacy contaminants ^a					
PAHs	X			X	X
PCBs	X	X	X		X
Heavy metals	X	X	X	X	
Organochlorine pesticides		X			
Chemical of concern measured during study ^b					
PAHs	5	2	3	7	6
Pesticides	2	0	0	1	3
Flavors/Fragrances	2	1	0	2	1
Plasticizers/flame retardant	3	1	0	4	6
Pharmaceuticals	1	0	0	5	2
Hormones	3	0	0	3	1
Total Detections	16	4	3	22	19

^aChemicals historically identified as contributing to the beneficial use impairments.

^bNumber of detects (concentrations above the laboratory detection limit) of chemical groups measured in water samples from Areas of Concern as described in Lee et al. [2012] and Choy et al. [2017].

not included. The sites did vary in the types of compounds identified in water and number of detects (Table V). The Ashtabula and Genesee sites had the lowest number of detects, while the St. Louis and Milwaukee had the highest, with Detroit just slightly lower (Table V). However, no trends could be discerned regarding particular chemicals and the prevalence/severity of MN or NA.

Brown bullhead from the Ashtabula and Detroit rivers had a low prevalence of MN but relatively higher prevalence of NA. Conversely, BB from the Genesee had a low prevalence of NA and higher prevalence of MN. Similar differences were seen in WS which had a higher prevalence of MN at the St Louis site and a lower prevalence of NA when compared to the Milwaukee site. This suggests exposure to a different suite of contaminants may be occurring at these sites as previous studies have shown certain chemicals may induce NA but not MN. For instance, experimental exposures to the herbicide pendimethalin [Ahmad and Ahmad, 2016], cyclophosphamide and cadmium [Ayllon and Garcia-Vazquez, 2000] and cadmium or copper [Güner and Muran, 2011] resulted in increased NA but not MN. Further chemical analyses are needed to identify the contaminants contributing to the observed results.

When comparing results obtained in the spring and fall there were no statistically significant differences in any of the parameters for either BB or LMB. However, in all cases, the values for MN and NA parameters were higher in the spring, with the exception of the reference site BB which had no MN in either season (Table II). This may indicate higher contaminant concentrations often associated with spring runoff events or perhaps seasonal physiological differences such as a more rapid turnover of erythrocytes as the water temperature rises in the spring.

Perhaps the most interesting results were the species differences in the occurrence and severity of erythrocyte

abnormalities, tumor prevalence and transcript abundance. Consistently, across sites, the bass or pelagic, top predator species had a higher prevalence of erythrocyte abnormalities than either WS or BB, the benthic-oriented species. Previous investigators have also demonstrated species differences in erythrocyte abnormalities and identified species that are less sensitive and so less useful as indicators through both field and laboratory exposures. For instance, Rodriguez-Cea et al. [2003] found minnows *Phoxinus phoxinus* and adult European eels *Anguilla Anguilla* did not demonstrate significant induction at contaminated sites while brown trout *Salmo trutta* did. The frequency of erythrocyte abnormalities within a cell population is dependent on the kinetics of cell proliferation [Al-Sab and Metcalfe, 1995] and consequently differences in metabolic rate and erythropoiesis can partially explain species differences [Soldatov, 2005]. Other factors such as diet and exposure pathways may also play a role. Grisolia et al. [2009] reported that piscivorous species showed the highest level of MN, while an omnivorous species had the highest frequency of NA. Interestingly, DNA damage as assessed by the comet assay (detects early DNA damage) was highest in the bottom feeding omnivorous species. These authors speculated that the findings may be associated with bioaccumulation of certain contaminants resulting in higher tissue contaminant levels in top predators, as well as feeding habits.

Another explanation for species differences in MN or NA frequency may be related to DNA repair or other mechanism associated with resistance or tolerance to chemical contaminants. The comet assay is another commonly used indicator of exposure to genotoxic compounds in the aquatic environment [Mitchellmore and Chipman, 1998; Lee and Steinert, 2003]. It detects DNA strand breaks, one of the first events after exposure to genotoxic contaminants and a potentially repairable

lesion, while MN and NA are non-repairable, clastogenic and aneugenic lesions [Frenzilli et al., 2009]. While low and non-significant differences in MN/NA of BB between sites have been noted [Metcalf, 1988; Smith, 1990], the comet assay has been successfully used in this species. Pandrangi et al. [1995] found the comet assay was a sensitive indicator of exposure in BB and able to detect differences between reference sites and sites contaminated with PAHs and PCBs. Yang et al. [2006] compared BB collected from the Ashtabula and Conneaut in spring 2004 and found significantly higher DNA damage at Ashtabula. They also found the greater DNA damage was associated with a higher prevalence of raised external lesions.

Neither skin nor liver tumors were observed in bass but were observed at all sites in WS or BB. No correlation between erythrocyte MN/NA and skin or liver neoplasms was observed. This supports previous research by Smith [1990], noting that although differences in liver neoplasm prevalence were observed between impacted and non-impacted sites, MN data yielded no significant difference among sites. Liver neoplasms were most prevalent at sites known to exhibit high sediment concentrations of aryl hydrocarbons, DDTs, and/or PCBs. Similarly, low levels of MN and NA were observed in BB injected with the genotoxic agents ethyl methanesulphonate or benzo(a)pyrene [Metcalf, 1988]. It is interesting to note that in the current study the pattern of bass MN severity among sites (Fig. 3B) was the same pattern as neoplasm prevalence in the benthic species (Fig. 5).

A number of benthic fish populations have been identified that exhibit a high prevalence of liver tumors but appear to be resistant to the more acute and/or toxic chemical effects. These include Atlantic tomcod *Microgadus tomcod* from the Hudson River and killifish or mummichog *Fundulus heteroclitus* from three Atlantic coast estuaries [reviewed by Wirgin and Waldman, 2004]. Mechanistically this resistance has been shown to be associated with the *ahr* variants [Wirgin et al., 2011; Reid et al., 2016] together with reduced expression of *cyp1a* [Wirgin and Waldman, 2004] suggesting the *ahr* pathway is less sensitive to activation in resistant populations [Yuan et al., 2006]. The *ahr* pathway is activated when ligands enter a cell and bind to the receptor which exists in an inactive state as a multiprotein complex with a number of proteins including *hsp90* [Denison et al., 2011]. The activation of *ahr* induces expression of Phase I xenobiotic-metabolizing enzymes such as *cyp1a* which hydroxylate hydrophobic substances such as PAHs and PCBs making them more amenable to Phase II metabolism by enzymes such as *gst* [Paetzold et al., 2009]. While *ahr*, *hsp90b* and *gst* transcripts were higher in the benthic fish, *cyp1a* was not higher in BB when compared to LMB (Tables III and IV). Williams and Hubberstey [2014] evaluated *cyp1a* and *p53* in BB captured at reference versus contaminated sites in Lake Erie and

suggested that a high expression of *p53* and a low expression of *cyp1a* may be an adaptive response in the populations from contaminated sites.

Oxidative stress is an important mechanism for both genotoxic and carcinogenic effects and *cyp1a* can play a role in the metabolic activation through oxygen radical formation and other mechanisms [Williams et al., 1998; Mena et al., 2009]. When combining the transcript data from the AOC sites, BB had significantly higher transcripts of *gpx*, *gst* and *sod*, enzymes commonly associated with oxidative stress, compared to LMB, as did WS compared to SMB (Tables III and IV). Studies on a population of mummichogs at a creosote contaminated site have shown that while they are resistant to the more acute/lethal effects they do develop hepatic neoplasms. One suggested mechanism for this resistance was elevated *gst* [Armknrecht et al., 1998]. Weis [2002] also reported the upregulation of *gst* along with non-inducible *cyp1a* among contaminant-resistant mummichog at contaminated sites. Conversely, an upregulation of hepatic *cyp1a1* and *gst* was observed in multixenobiotic-resistant mummichogs [Paetzold et al., 2009].

In conclusion, the results demonstrate the utility of MN and NA as additional biomarkers to assess ecosystem health at Great Lakes AOCs, as well as the importance of assessing multiple species and endpoints. While the bass species exhibited a higher prevalence of genotoxic response as indicated by MN and NA, the benthic species had a higher prevalence of skin and liver neoplasms and higher gene transcripts for key enzymes induced by contaminant exposure. Taken together these findings suggest while both fish species at a site are exposed to genotoxic contaminants, BB and WS may have evolved enhanced DNA repair or other mechanisms of contaminant resistance after decades of exposure. Conversely, the data may indicate that factors other than genotoxic chemicals contribute to the observed liver and orocutaneous neoplasms. Further analyses of water, sediment and tissue contaminants, together with biological indicators will be necessary to better elucidate the mechanisms behind these differences.

AUTHOR CONTRIBUTIONS

RPB, VSB, and CHS participated in collection of samples, sample analyses and analysis and interpretation of the data. RPB and VSB wrote the manuscript with assistance from CHS and PMM. PMM also provided administrative oversight and served as the major professor for RPB. All authors read and provided input in finalizing the manuscript.

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